X Chromosome Inactivation Pattern is not Associated With Interindividual Variations in Thyroid Volume: A Study of Euthyroid Danish Female Twins

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A higher frequency of skewed X chromosome inactivation (XCI) is found in patients with autoimmune thyroid disease (AITD) than in controls. Although goitre is often present in AITD, a recent study failed to show an association between XCI and clinically overt nontoxic goitre. However, the etiology of overt goitre is complex, and the mechanisms influencing thyroid volume may involve fewer factors than the mechanisms underlying overt goitre. In order to examine the impact of XCI on thyroid volume in euthyroid females, we studied whether within cohort (n = 138) and within twin pair (n = 69) differences in XCI are correlated with differences in thyroid volume. XCI was determined by PCR analysis of a polymorphic CAG repeat in the first exon of the androgen receptor gene. Thyroid volume was determined by ultrasound. Neither in the within cohort nor in the within twin pair analysis could we demonstrate a statistically significant association between XCI and thyroid volume: Regression coefficient (β) = 0.023 (95% confidence interval, –0.062 –0.108), p = 0.592 and β = 0.038 (–0.080–0.156), p = 0.521, respectively. Controlling for potential confounders such as zygosity, age, TSH, smoking habits and use of oral contraceptives did not change the findings. In conclusion, in a sample of euthyroid Danish female twins, we found no evidence of a relationship between XCI pattern and thyroid volume.

Keywords: X chromosome inactivation, thyroid volume, twins, epigenetics

Thyroid diseases are among the most common disorders in the general population. Using ultrasound, as many as 50% of individuals harbour one or several nodules within their thyroid gland (Wang & Crapo, 1997). As many as 15% have a palpable goitre, 10% demonstrate an abnormal serum concentration of thyrotropin (TSH), and up to 5% of women have overt hypo- or hyperthyroidism (Laurberg et al., 2001; Vanderpump et al., 1995; Wang & Crapo, 1997). Despite this high frequency, the etiology of these disorders is incompletely understood. However, it is generally assumed that the development of clinically overt thyroid disease is the net result of a number of environmental exposures — especially the level of iodine intake (Laurberg et al., 2001) and cigarette smoking (Brix et al., 2000a) operating in genetically predisposed individuals (Brix et al., 1999; Brix et al., 2000b; Brix et al., 2001). In addition, constitutional factors such as sex, are clearly involved in the etiology of thyroid disease, because the ratio of females to males for most phenotypes exceeds 5:1 (Vanderpump et al., 1995; Wang & Crapo, 1997).

This phenomenon of female predisposition to overt thyroid disease is often ascribed to hormonal differences, because in experimental disease models, estrogens stimulate whereas androgens inhibit growth of thyroid cells/disease activity (Kawabata et al., 2003; Manole et al., 2001). However, studies in man have failed to demonstrate a clear-cut influence of sex hormones on thyroid disease (Barrere et al., 2000; Knudsen et al., 2002). Moreover, the observed gender difference in thyroid disease and other common diseases extends far beyond the hormonal differences (Ober et al., 2008). Recent evidence indicates that some of the observed differences between males and females in the prevalence of many common diseases might also be due to sex-specific differences in genetic architecture (Ober et al., 2008).

Theoretically, a skewed X chromosome inactivation (XCI) pattern and resultant tissue chimerism could offer a novel explanation for the female preponderance of

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thyroid disease. In female mammalian cells, one of the two X chromosomes is inactivated in early embryonic life in order to equalize the dosage of X linked genes between females and males (Lyon, 1961). This process is random and permanent for all descendants of a cell (Brown & Robinson., 2000). Thus, females are mosaics for two cell lines, cells with the paternal and cells with the maternal X chromosome as the active X. Most females have approximately 50% (random distribution) of each cell line (Kristiansen et al., 2005). A skewed XCI pattern represents a marked deviation from this distribution. In addition, the X chromosome may be of particular interest in thyroid disorders because a number of genetic markers linked to autoimmune thyroid disease (AITD) (Barbesino et al., 1998; Imrie et al., 2001) and goitre (Capon et al., 2000) are X-linked. It follows that a skewed XCI could result in expression of X linked diseases in females due to predominant inactivation of the normal X chromosome.

Recently, we (Brix et al., 2005) and subsequently others (Ozcelik et al., 2006; Yin et al., 2007) suggested a skewed XCI to be involved in the etiology of AITD. Although goitre is often present in AITD, a recent study failed to show an association between XCI and clinically overt non-toxic goitre (Brix et al., 2009). However, in that study (Brix et al., 2009) the information regarding presence or absence of goitre was based on self-reports. Clearly, this will result in some degree of recall bias and therefore some misclassification of both cases and controls may have occurred, hampering the interpretation.

Moreover, the etiology of overt goitre is complex, and the mechanisms influencing thyroid volume may involve fewer factors than the mechanisms underlying overt goitre. Consequently, we have examined the relationship between thyroid volume determined by ultrasound and XCI in a cohort of 138 euthyroid female twin individuals.

Subjects

The twins were recruited from the The Danish Twin Registry (Skytthe et al., 2006). This study is part of a nationwide project (GEMINAKAR) investigating the relative influence of genetic and environmental factors on insulin resistance, obesity and cardiovascular risk factors in self-reported healthy twin pairs. A detailed description of the ascertainment procedure in the GEMINAKAR project has been published elsewhere (Benyamin et al., 2007). In brief, in 1997 a representative sample of self-reported healthy twin pairs born between 1931 and 1982 was recruited from the Danish Twin Register on the basis of nationwide questionnaire surveys concerning health and health related behavior performed in 1994 and 1996. In all, 1512 individuals (756 twin pairs) were examined from 1997 to 2000. Blood samples were available from 736 twin pairs. Twin pairs with self-reported thyroid disease (32 subjects in 28 twin pairs) or overt biochemical thyroid disease (19 subjects in 18 pairs) were excluded. Moreover, all males (688 subjects) and females from opposite sex pairs (120 subjects) were also excluded leaving 572 female subjects (286 pairs). Of these, 138 subjects (69 twin pairs, distributed in 37 monozygotic (MZ) and 32 dizygotic (DZ) pairs) were informative regarding both thyroid volume and XCI pattern and hence suitable for data analysis. The study was approved by all the Regional Scientific-Ethical Committees in Denmark (case file. 97/25 PMC).

Methods

Assays

X-Chromosome Inactivation Analysis

DNA was extracted from peripheral blood cells. The X chromosome phenotype was determined by polymerase chain reaction (PCR) analysis of a polymorphic (CAG)n repeat in the first exon of the androgen receptor gene (Allen et al., 1992). After digestion of the DNA with the methylation sensitive enzyme *Hpa*II, a PCR product is obtained from the inactive X chromosome only. The PCR products were separated on an ABI 3100 automated sequencer, and analyzed by GeneScan software (Applied Biosystems, Foster City, California, USA). Each sample was analyzed in duplicate and blinded as to the result in the corresponding co-twin. XCI was calculated as the percentage of the predominantly inactive allele to the sum of both alleles and varies between 50 and 100, where 50 reflects random XCI and 100 reflects a completely skewed XCI.

Serum TSH and Zygosity Determination

Serum concentrations of TSH were measured using a solid-phase time-resolved fluoroimmunometric assay (AutoDELFIA, Perkin-Elmer / Wallac, Turku, Finland). The reference range for TSH is 0.58–4.07 mU/liter (Jensen et al., 2004). All serum samples were analyzed at the same laboratory, and twin individuals from the same pair were analyzed within the same run.

Zygosity was established by analysis of nine highly polymorphic restriction fragment length polymorphisms and micro satellite markers scattered widely throughout the genome with a PE Applied Biosystems AmpFISTER Profiles Plus Kit (Foster City, California, USA).

Thyroid Volume

Thyroid volume was evaluated on the basis of an ultrasonic scanning procedure using a 5.5-MHz compound scanner (type 1846, Brüel and Kjær, Naerum, Denmark). The estimation of thyroid volume was based on recordings of cross-sectional areas through the gland at 0.5 cm intervals, followed by computerized calculation of the volume (Hegedüs et al., 1983). For each twin pair, the volume measurement was performed by the same operator (LH, FNB or SJB) with blinding toward zygosity and thyroid volume of the co-twin.

Statistical methods

Group frequencies were compared with the Pearson χ^2 test, whereas group medians were compared with a Mann-Whitney test using Brunner's adjustment for clustering within twin pairs (Brunner, 1991). The relationship between XCI and thyroid volume (within

cohort analysis) as well as the relationship between the within twin pair differences in XCI and within pair differences in thyroid volume (within twin pair analysis) were assessed by linear regression.

In the within-cohort analysis, the paired nature of the twin data was taken into account by using the cluster option in STATA (StataCorp, College Station, Texas, USA). Subsequently, the data were analyzed with thyroid volume as the response variable and XCI, TSH, age, smoking, use of oral contraceptives and zygosity as the explanatory variables. In the within twin-pair analysis, the regression line was constrained to pass through the origin so that the results were independent of the labeling of the twin as the first or second. Subsequently, we stratified according to zygosity and adjusted for serum TSH, smoking and use of oral contraceptives by using the within pair differences of these variables as explanatory variables in the regression model.

Significant differences were defined as a P-value less than 0.05 using two tailed tests. All analyses were carried out using version 7 of the STATA statistical package (StataCorp, College Station, Texas, USA).

Results

Descriptive Statistics (Table 1)

Descriptive characteristics in the twin population as a whole and stratified by zygosity are given in Table 1. A statistically significant difference between MZ and DZ twins was observed for smoking (MZ vs. DZ; 31% vs. 52%, p = .015) and age (MZ vs. DZ; 29 vs. 36.5 years, p = .002). There were no statistically significant differences in the XCI pattern, thyroid volume and serum TSH concentration across zygosity.

Table 1

Basic Characteristics

Variable	Study population		
	MZ + DZ (n = 138)	MZ (n = 74)	DZ (n = 64)
Smoking (%)	41	31[a]	52
Age (year)	34 (19–51)	29 (19–51)[b]	36.5 (21–52)
XCI pattern (%)	65 (52–87)	65 (51–86)[c]	64 (52–87)
Volume (mL)	13.9 (7.9–23.3)	13.5 (9.0–23.3)[c]	14.0 (7.5–23.1)
TSH (mU/liter)	1.39 (0.65–3.18)	1.53 (0.65–3.55)[d]	1.26 (0.61–2.23)

Note: All values, except for smoking, are presented as medians with 5th and 95th percentiles in parentheses. MZ = monozygotic and DZ = dizygotic.
[a] MZ vs DZ, p = 0.015; [b] MZ vs DZ, p = 0.002; [c] MZ vs DZ, p > 0.300;
[d] MZ vs DZ, p = 0.054

Regression Analysis (Figure 1)

The results of the within cohort and within twin pair analyses are outlined in Figure 1. Overall, irrespective of zygosity, no significant associations were found (Figure 1, A and B). Stratification for zygosity in the within twin pair analysis did not change the results (Figure 1, C and D). Controlling for potential confounders (age, serum concentration of TSH, smoking habits and use of oral contraceptives) did not change the findings of nonsignificant regression coefficients in the within cohort analysis, regression coefficient (β) = .037 (95% confidence interval, –.044–.117), P = .366) or in the within twin pair analysis, β= .059 (–0.064–0.181), P = .343. When

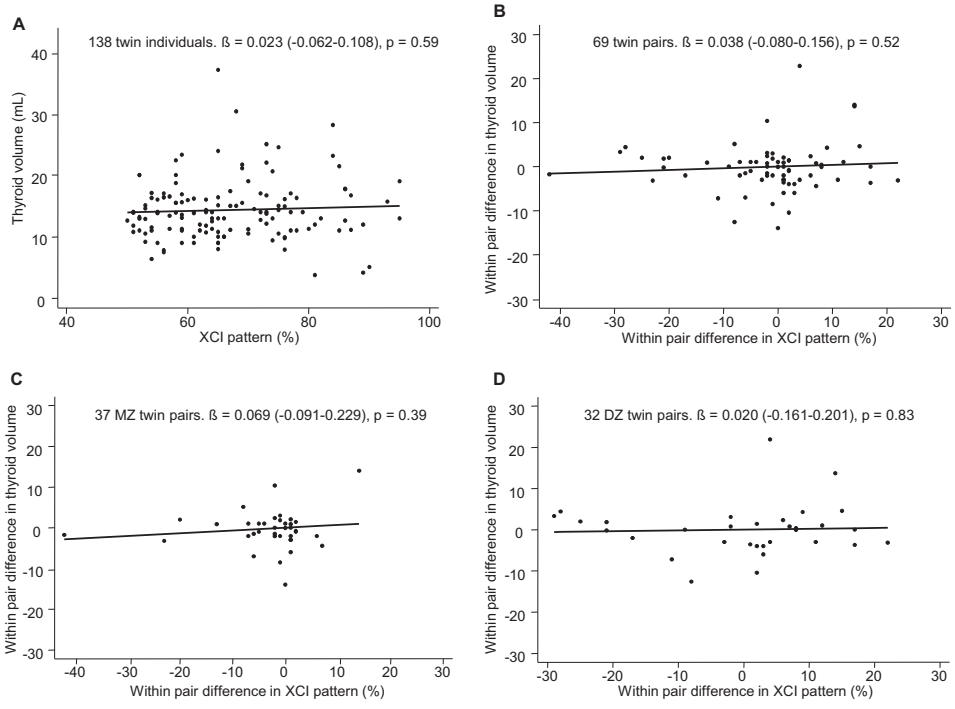

Figure 1

Scatterplots and unadjusted regression coefficients (ß) for the correlation between thyroid volumen and X chromosome inactivation (XCI) pattern for the within cohort (A) and within twin-pair (B, C and D) comparisons. Ninety-five per cent confidence intervals are given in parentheses.

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restricting the analyses to twin pairs with a within-pair difference in XCI of at least 10% the regression coefficient was almost unchanged: β = .026 (–.101–.152), *P* = .677. Essentially similar results were obtained when the analyses were restricted to twin pairs with a within pair difference in thyroid volume of at least 4 or 8 ml (data not shown).

Discussion

The reason for the female predominance of goitre, with or without thyroid dysfunction, remains largely unknown. The present study was initiated to test the hypothesis that a nonhormonal sex specific genetic mechanism related to XCI could influence thyroid volume in euthyroid female twins. According to the hypothesis, subjects with the largest thyroid volume should have the highest degree of skewed XCI pattern. In our sample of euthyroid Danish female twins, we did not observe a significant correlation between XCI pattern and thyroid volume. To minimize the effect of genetic and environmental confounding we also analyzed the association between XCI and thyroid volume within MZ and DZ twin pairs, which did not change the finding of a non-significant association. Essentially similar results were obtained when the analyses were restricted to twin pairs with large within pair differences in thyroid volume and XCI. Thus, neither in the within cohort nor in the within twin pair analysis could we demonstrate a statistically significant association between XCI pattern and thyroid volume, extending our previous findings in overt goitre (Brix et al., 2009).

The strengths of this study include; a relatively large sample size (*n* = 138), ascertainment of participants from a nation-wide population based register, use of a very precise method for determination of thyroid volume, and exclusion of subjects with evidence of thyroid disease. In addition, our study is very robust, because the twin design allows optimal control for genetic and environmental factors affecting both thyroid volume and XCI pattern (MacGregor et al., 2000).

All the participants were twins, but there is no reason to suspect that the XCI process and the factors influencing thyroid volume differ between twins and singletons. Available studies have clearly shown that Danish twins are representative of the general background population for a range of thyroid phenotypes including goitre (Brix et al., 1999), thyroid size (Hansen et al., 2004), as well as XCI pattern (Kristiansen et al., 2005). It is well known from epidemiological surveys that the spectrum of thyroid disease, especially goitre related phenotypes, in a community changes with variations in the iodine intake (Laurberg et al., 2001). In the present study, all participants were Caucasian women living in Denmark, an area with borderline iodine deficiency and with only minor regional differences in iodine intake. Moreover, cultural background and living conditions are generally quite homogeneous in Denmark. Consequently, our results cannot uncritically be extrapolated to other populations. Finally, XCI was tested in peripheral blood and not from the thyroid gland. Understandably, thyroid biopsies were not available from these healthy subjects.

In conclusion, in a sample of euthyroid Danish female twins, we found no evidence of a relationship between XCI pattern and thyroid volume.

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Disclosure statement

Thomas Heiberg Brix, Pia Skov Hansen, Finn Noe Bennedbæk, Steen Joop Bonne-ma, Kirsten Ohm Kyvik, Karen Helene Ørstavik and Laszlo Hegedüs state that they have no conflicts of interest.

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